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Abstract
The digestibility of plant biomass has a major influence on its value as a forage for livestock and as a feedstock for industrial biotechnology. For both processes, the concentration, structure, and composition of lignin influence the accessibility of wall carbohydrate polymers to microbes and digestive enzymes during biochemical decomposition. Although lignin engineering has been less tractable in monocots than in model dicots, a body of work is accumulating on the effects of manipulating lignin biosynthesis in energy grasses and cereal crops. In addition to conventional targets for lignin engineering, several novel features of grass lignin have recently become amenable to targeted manipulation through the identification of genes involved in their synthesis.

Introduction

Many feedstocks that might be used for future biorefinery applications come from grasses, be it biomass from perennial energy crops (switchgrass, miscanthus, sugarcane) or agricultural coproducts such as cereal straw from food crops. In these materials, lignin can represent 20% or more of the biomass, and will inhibit biomass digestion in several ways, mainly by preventing microbes and enzymes from gaining access to cellulose, but also by binding digestive enzymes or releasing inhibitory breakdown products. Physical and chemical pretreatments are therefore necessary to facilitate biomass digestion by removing some xylans and lignin to enable enzymes to gain access to the hydrophobic cellulose face [1] and release simple sugars (saccharification). An ideal pretreatment might remove as much lignin as possible while minimizing polysaccharide modification to retain a near-native microfibrillar structure that enzymes can attack [1]. Ruminant animals have solved the problem of releasing sugars from plant biomass in ways somewhat analogous to industrial biochemical processing, i.e., by effective physical pretreatment (chewing, and rechewing regurgitated solids), then slow microbial fermentation in the rumen and peptic/enzymatic digestion of non-fermented material. Although biomass digestion in the rumen, and in industry, may not always depend on the same factors, there is sufficient commonality for synergy between both camps in identifying targets to increase biomass digestibility.

Special features of lignin biosynthesis in grasses

Lignin biosynthesis and structure are discussed elsewhere in this issue [2,3]. Briefly, lignin is a polymer of predominantly three monolignols *p*-coumaryl alcohol, coniferyl alcohol, and sinapyl alcohol, giving rise to H, G and S units. Like dicots, grasses have an SG-type lignin but with significantly more H units, although H levels are still low. The enzymes of the phenylpropanoid pathway that produce these monolignols are obvious targets for lignin genetic engineering to improve biomass digestibility (Figure 1). This ‘conventional’ lignin pathway operates similarly in dicots and grasses with a few distinctions. A key intermediate, *p*-coumarate, derives partially from deamination of tyrosine in grasses, whereas it derives solely from deamination of phenylalanine followed by 4-hydroxylation in dicots. Some grass PAL enzymes also possess tyrosine ammonia-lyase activity and are bifunctional phenylalanine/tyrosine ammonia-lyases, PTALs [4,5]. In addition, CSE is crucial to lignin biosynthesis in several dicots and influences saccharification efficiency but only some grasses have an orthologous gene [6-8].

In addition to conventional components, grass lignins have several special features that offer targets for lignin engineering beyond those usually possible in dicots (Figure 1). These include the incorporation of the flavone tricin as an additional lignin monomer [9,10], the *p*-coumaroylation of (mainly) S lignin units, and the incorporation of ferulates into lignin [11]. Genes controlling incorporation of these components are only now coming to light and present novel lignin engineering opportunities. Because of the clear differences in structure between grass and dicot lignins, the results of specific gene manipulations in dicots should not be extrapolated to possible digestibility benefits in grasses – this must be evaluated directly in grass species.

Conventional targets for reducing lignin content in grasses

It is absolutely clear that the amount of lignin in grass cell walls is generally negatively correlated with digestibility. For example, in maize inbreds, diverse miscanthus species and a variety of cereal crops, biomass digestibility in rumen fluid or glucose yields on saccharification were negatively correlated with Klason lignin [12-14]. To reduce lignin content, engineering strategies can be targeted at the early phenylpropanoid pathway to alter flux into all subsequent steps, i.e., manipulating PTAL and 4CL. Manipulating C4H is unlikely to be effective as grasses can bypass a requirement for C4H using PTAL (Figure 1). When multiple *PAL* genes were suppressed by RNAi in brachypodium the result was a large 43% decrease in lignin and nearly 2-fold increase in sugars released after a limited-extent digestion [4]. Increases in S/G and H units were explained by potential differences in relative flux through those arms of the pathway. However, the plants showed developmental delays and increased susceptibility to some pathogens [4]. More specific suppression of *PTAL* would be instructive but, although such plants have been produced [5], digestibility data has not been reported.

Suppression of class I *4CL*s (i.e., *4CL*s potentially involved in monolignol biosynthesis) in switchgrass, rice, sugarcane and sorghum *bmr2* mutants leads to around 17-20% lignin reduction, usually with brown midrib-like phenotypes [15-18]. In cases in which lignin monomer ratios were determined, these generally showed equivalent reductions in G and S, although switchgrass showed differential effects on the three monolignols. Biomass yield was normal in most cases although rice plants were shorter and less fertile than controls [16]. Sugarcane had reduced height but produced more tillers so biomass was maintained in most lines, even in the field [17]. Saccharification efficiency after dilute acid pretreatment was 52-76% improved in 4CL-suppressed switchgrass and field-grown sugarcane [15,17]. Thus it appears that 4CL can be an effective target for moderate reduction of lignin content with significant benefits for digestibility even in field-grown plants.

Genes common to all three branches of the lignin pathway (i.e., *CCR* and *CAD*) might also be expected to reduce overall lignin content. This has proven to be the case although changes to monomeric ratios have also been seen. Digestibility improvements consequent on CCR deficiency (by RNAi or mutation) have been reported in maize and perennial ryegrass [19-22]. Lignin content was lowered by 9-37%, with all three monomers generally reduced (although results on H units were inconsistent between maize studies), and S/G ratio either not altered or increased. Both species showed no change to growth, even in field-grown plants, and digestibility in rumen fluid was 14-15% improved [19,20]. Glucose release after pretreatment and limited saccharification increased by up to 40% in maize. As with *CCR*, *CAD*-

suppression should affect all three monolignol branches. Mutants or transgenics for the major lignin biosynthetic CAD exist in many grasses and consistently show 6-26% reductions in lignin [23-30]. Impacts on G and S vary, possibly reflecting a contribution of other *CAD* genes, or differences between *CAD* knock-out mutants and knocked-down/suppressed plants. S units are generally the most reduced component, with G units reduced or proportionally increased in different cases, generally resulting in a drop in S/G [24-26] or no S/G change [23,27,29]. Sinapaldehyde [24,26] and sometimes coniferaldehyde [29] is present in the lignin which may have a more condensed structure. An enrichment in lignin free-phenolic end-groups makes the polymer more soluble in alkali. Both 44-46% increased sugar yields after limited saccharification [26], and increased ethanol yields [28] are reported after mild alkaline pretreatments. Digestibility in rumen fluid is also increased [24,29].

Conventional targets for altering lignin monomer ratios in grasses

Studies conflict on whether changes to lignin monomer ratios could have benefits for biomass digestibility. Differences between experiments in species studied, growth conditions, tissue harvested, lignin analysis method, pretreatment used, etc., likely influence results and make it difficult to draw conclusions. Illustrative experiments in which both lignin compositional data and digestibility are reported are discussed below to enable some evaluation.

The proportion of H units in lignin might be increased by suppressing enzymes that divert *p*-coumaroyl-CoA into other branches, i.e., HCT, C3H and CSE. To date, only the first two have been manipulated in grasses. Maize RNAi-suppressed in one of two *C3H* genes had the proportion of lignin H units increased from 2% to nearly 8% with little change in overall lignin levels; one line had improved biomass degradability but growth was affected [31]. Rice CRISPR/Cas9-mediated *C3H* knockouts arrested development at the 4-5 leaf stage, although less severely affected *C3H*-RNAi-suppressed plants had only slightly reduced height [32]. *C3H*-RNAi lines had reductions in stem total lignin, G, S and ferulate units, with increased levels of β-ether-linked H units, tricin and *p*-coumarate [32]. Most likely several of these changes contributed to the observed improved saccharification yields [32]. Nevertheless, the consistent appearance of severe phenotypes, both in grasses and in dicots, when *HCT* or *C3H* are suppressed, suggest that they are not robust targets for lignin engineering.

It has been suggested from dicot studies that S/G ratio influences digestibility. S/G might be specifically manipulated by targeting CCoAOMT, F5H (CAld5H) or COMT. Maize and rice have been produced with manipulated CCoAOMT or F5H, but digestibility was not reported [33,34]. By comparison, *COMT*, the gene underlying improved digestibility in *bm3* mutants, has been an obvious target for transgenic manipulation in biomass and forage grasses and cereal crops [e.g., 35-38] with several evaluations progressing to the field [38-42]. Together, these demonstrate changes in *COMT*-suppressed transgenics consistent with those in *bm3* plants, i.e., moderate reductions to lignin (6-14%), with more dramatic reductions in S units (20-46%) accompanied by a decrease in S/G. Some reports record reduced levels of ester-linked *p*-coumarate in lignin with normal levels of ferulate [37,40] and/or the appearance of ‘signature’ 5-OH-G units [35,37]. Growth is generally normal although field-grown sugarcane had normal biomass at 6% lignin reduction, but decreased biomass at 8-12% lignin reduction [40]. Digestibility for forage use was increased in

COMT-suppressed maize, ryegrass, and tall fescue by 9-11% [35,38,43]. Likewise, saccharification efficiency was increased in sugarcane and switchgrass under a range of conditions [40,41,44] and product yields on simultaneous saccharification and fermentation, or after fermentation with *Clostridium thermocellum* (capable of consolidated bioprocessing), were also increased [36]. Switchgrass transgenics had over 20% improvement in cellulose accessibility [44], likely a crucial factor in the improved digestibility. Similarly, *COMT* brown-midrib mutants are thought to be more digestible only because of their lower lignin content, the modified lignin being more inhibitory to biomass digestion [45].

Novel targets for lignin engineering in grasses

Given that tricin is a lignin monomer and appears to function as a nucleation site for lignification in grasses [46,47], inhibiting such nucleation might reduce lignin content and increase digestibility. However, a maize mutant in *chalcone synthase C2* (acting at the entry point to the flavonoid pathway) with dramatically reduced tricin had increased leaf lignin due to redirection of flux from the blocked flavonoid pathway into lignin, reducing leaf saccharification efficiency [48]. COMT is involved in later steps in tricin biosynthesis and its suppression led to reduced tricin [37,49,50]; conversely suppression of C3H in rice increased tricin [32], yet both types of manipulation improved saccharification. A rice mutant in the *FNSII* gene specific to the flavonoid pathway and devoid of tricin had enhanced saccharification efficiency, but also unexpectedly showed reduced lignin S/G, as well as incorporating the intermediate naringenin in place of tricin [51]. Given the range of changes to lignin in these variously tricin-modified plants, improved saccharification cannot be assigned to any specific change. Clearly, the interactions between flavonoid and lignin biosynthesis require more study before they can be manipulated in a predictable way.

The *p*-coumarate in grass lignin mainly acylates S units; it is attached to monolignols intracellularly by a BAHD *p*-coumaroyl-CoA:monolignol transferase or PMT [52-55]. Suppression and overexpression of *PMT* in brachypodium resulted in 10% decrease and 3-fold increase respectively in *p*-coumarate on lignin. *PMT*-overexpressing plants gave higher sugar yields on saccharification after certain pretreatments, although they also had reduced Klason lignin and changes to lignin monomer ratios [53]. Similarly, brachypodium *PMT* expressed in arabidopsis under the control of the C4H promoter produced levels of lignin coumaroylation comparable to those in grasses, decreased lignin content by 10-30%, and increased saccharification yields with and without pretreatment [54]. The greatest saccharification improvements were seen in an Arabidopsis *CCR*-mutant background in which lignin ferulate esters were also increased; these plants had a higher incidence of lignin free-phenolic end-groups with 10% decreased lignin and released about 50% more glucose on saccharification [54].

The presence of ferulates is another interesting phenomenon of grass cell walls. Quite high levels acylate arabinoxylans and produce important cross-links between arabinoxylan chains and between arabinoxylans and lignin [55]. The chemistry of such cross-links is reviewed elsewhere in this issue [56] and their presence in cell walls could clearly impact wall pore-size and the mobility of cell wall-digesting enzymes. Several *BAHD* genes have been implicated in the conjugation of ferulate to xylan's arabinosyl units as their downregulation reduces wall-bound ferulate in rice [57], brachypodium [58] and *Setaria* [59]. In the later case, a significant improvement in saccharification efficiency was also demonstrated. The changes in these plants

appear relatively specific to ester-linked ferulate reduction as lignin content was not altered, although there were increases in wall-esterified *p*-coumarate. It was recently discovered that ferulates also acylate monolignols, primarily sinapyl alcohol in grasses, and are incorporated into lignins to produce so-called 'zip-lignins' [60]. *BAHD* genes related to those known to conjugate ferulate to arabinoxylan are likely involved in conjugating ferulate to monolignols. A rice feruloyl-CoA monolignol transferase (FMT) has been identified and its overexpression in activation-tagged mutants or transgenics increases β-ether-bonded monolignol ferulates [60]. Such conjugates were also increased in maize CCR-deficient mutants in which the flux of feruloyl-CoA toward G and S lignin biosynthesis is blocked [21], adding yet another change to the lignin modifications described in CCR-deficient plants that may contribute to improved saccharification sugar yields.

Conclusions

Unravelling the influences on digestibility in grasses of different components and structures in lignin is difficult, as several features concomitantly change in most genetic interventions, illustrating inter-dependency between them. A constant feature in many improved-digestibility transgenic grasses and mutants is a moderate reduction in lignin. This has no, or minimal, impacts on biomass yield for several gene targets (*4CL*, *CCR*, *COMT*, *CAD*, *PMT*). Other lignin changes seen in several but not all improved-digestibility transgenics include reduced or unaltered S/G, an increase in free-phenolic end-groups, and possibly a decrease in esterified *p*-coumarate. These generalisations need to be viewed with caution given the small number of underpinning experiments, and the lack of digestibility data for several potential targets. Adverse effects on growth, if they occur, might be mitigated by careful screening of transgenic events and by introgressing into several cultivars to explore the most compatible background genetics. In addition, the advent of gene editing enables more precise and stable engineering of specific gene/enzyme activity levels to help avoid adverse effects. The several experiments and targets that have now progressed as far as field trials and still show beneficial effects of lignin engineering for digestibility, should encourage optimism for the future and the exploration of conventional and novel targets in increasingly sophisticated ways.

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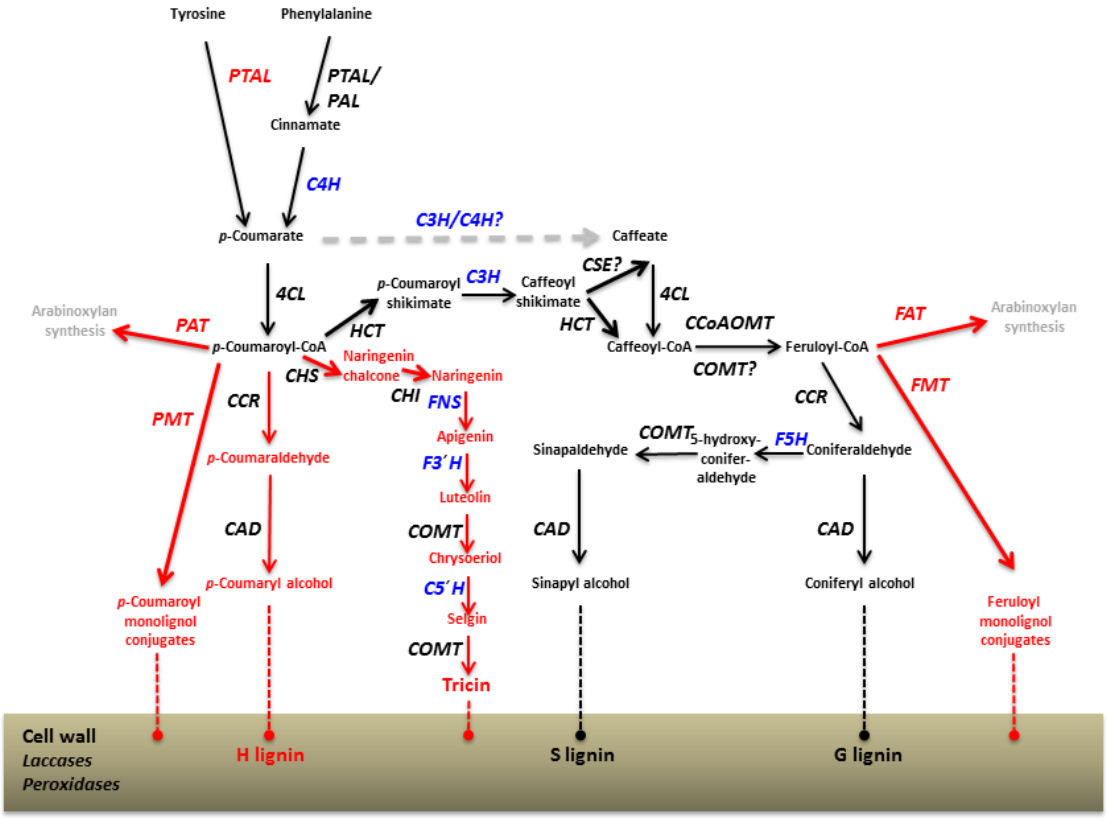
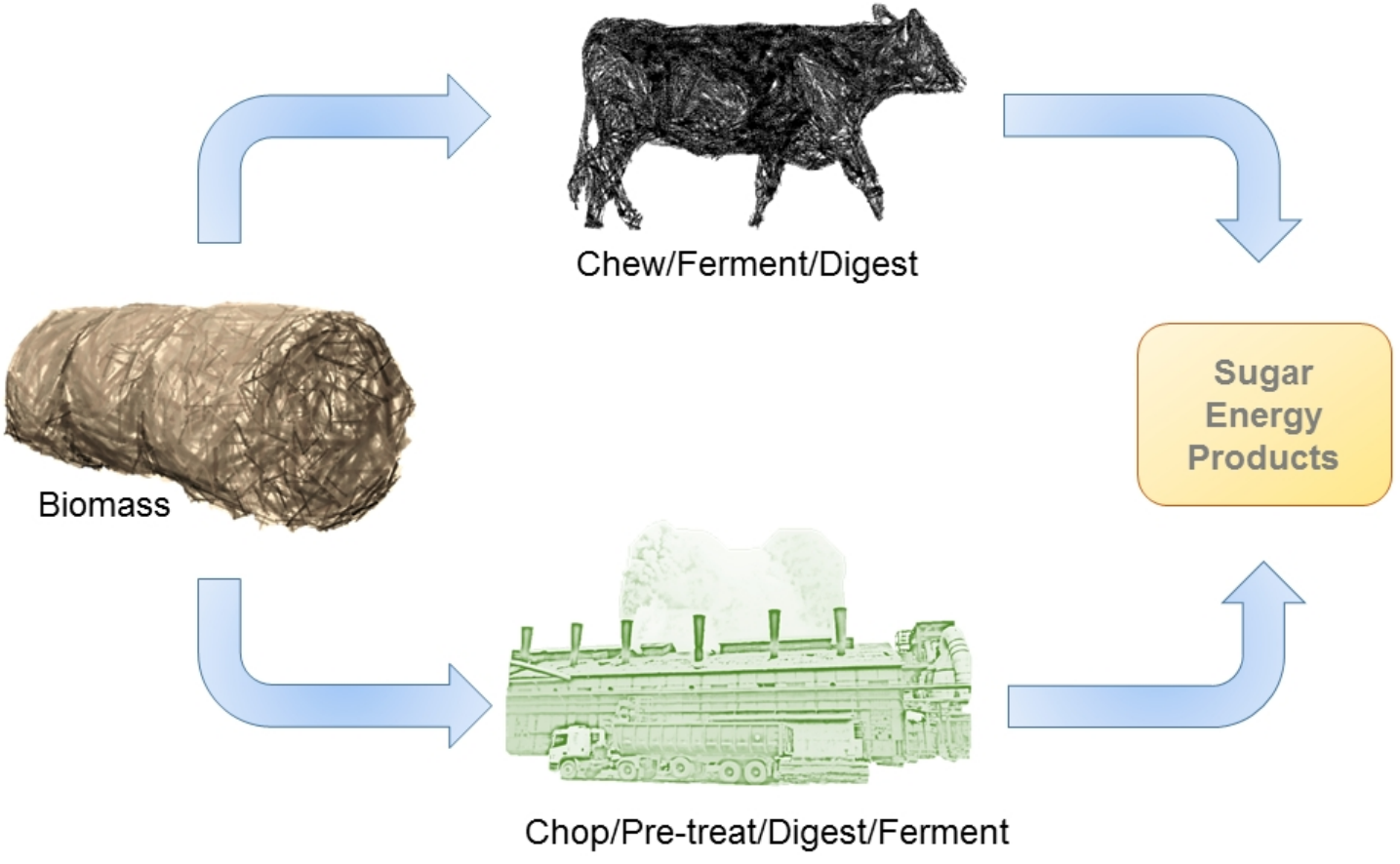
Demonstration that rice OsAT5 and other grass orthologues are likely BAHD feruloyl-coenzyme A monolignol transferases (FMTs) and a survey of the presence of monolignol ferulate in lignins among plant species.

Figure 1. Lignin biosynthesis in grasses. Most likely routes to intermediates are shown but do not preclude other routes. Only abbreviated enzyme names are used in the text. Red arrows denote pathways of specific importance in grasses. Blue enzyme/gene names denote membrane-bound P450 enzymes that may be in close proximity to each other.

PAL - phenylalanine ammonia lyase; PTAL - bifunctional phenylalanine:tyrosine ammonia lyase; C4H - cinnamate-4-hydroxylase; 4CL - 4-coumarate:CoA ligase; HCT - *p*-hydroxycinnamoyl-CoA:quinate/shikimate *p*-hydroxycinnamoyltransferase; C3H - *p*-coumarate 3-hydroxylase; CSE - caffeoyl shikimate esterase; CCoAOMT - caffeoyl-CoA *O*-methyltransferase; COMT - caffeate *O*-methyltransferase; CCR - cinnamoyl-CoA reductase; CAD - cinnamyl alcohol dehydrogenase; F5H - ferulate 5-hydroxylase; PAT - *p*-coumaroyl-CoA arabinoxylan transferase; PMT - *p*-coumaroyl-CoA monolignol transferase; FAT - feruloyl-CoA arabinoxylan transferase; FMT- feruloyl-CoA monolignol transferase; CHS - chalcone synthase; CHI - chalcone isomerase; FNS - flavone synthase; F3´H - naringenin 3-dioxygenase; C5´H - chrysoeriol 5-hydroxylase

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